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Studies on the endothelin-1-induced contraction of rat granulation tissue pouch mediated by myofibroblasts

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Abstract

A granulation tissue pouch, mostly composed of myofibroblasts, was prepared by injecting rats subcutaneously with croton oil. The contraction of the granulation tissue pouch caused by endothelin-1 (ET-1) and the effects of the ET receptor antagonists, BQ123 and BQ788 on it were thus examined. ET-1 produced contractions in a dose-dependent manner. Pretreatment with BQ123, an ET_A receptor selective antagonist, shifted the dose–response curve to the right, whereas pretreatment with BQ788, an ET_B receptor selective antagonist, showed little effect. IRL1620, an ET_B receptor selective agonist, did not cause any contraction in the granulation tissue pouch. The existence of both ET_A and ET_B receptors in the granulation tissue pouch and in cultured myofibroblasts was demonstrated by RT-PCR. Intracellular Ca^{2+} mobilization in Fura-2/acetyl-methoxy ester loaded cultured myofibroblasts isolated from the granulation tissue was also examined. ET-1 produced a transient increase in $[Ca^{2+}]_i$ followed by a sustained elevation of $[Ca^{2+}]_i$, whereas IRL1620 caused only a transient peak. These results suggest that the ET-1 induced contraction of granulation tissue is mainly mediated through the mobilization of Ca^{2+} from the extracellular space caused by stimulation with ET_A receptor.

Keywords: Endothelin-1; Myofibroblast; Calcium mobilization; Wound healing

1. Introduction

The wound healing process involves complicated interactions between numerous components, such as the extracellular matrix, cytokines, and specific cellular elements [1,2]. Although the contraction of granulation tissue is an important process in wound healing, the mechanism has not yet been elucidated. During the course of granulation tissue formation, fibroblasts proliferate and differentiate into contractile phenotypes which are characterized by the appearance of cytoplasmic microfilaments and cytoskeletal proteins such as α-smooth muscle actin (α-SM actin) and vimentin [3,4]. These fibroblasts are called myofibroblasts and play an important role in both wound contraction and healing [5].

Many substances (i.e. 5-hydroxytryptamine, bradykinin, histamine, angiotensin, vasopressin, epinephrine, norepinephrine, ET-1) which cause contractions of smooth muscle have also been reported to produce contractions of granulation tissue strips [6–8]. Among these agonists, Appleton et al. reported that the level of ET-1 synthesis displays a time course parallel to the extent of shrinkage of the wound.

Abbreviations: ET-1, endothelin-1; α-SM actin, α-smooth muscle actin; RT-PCR, reverse transcriptase-polymerase chain reaction; Fura-2/AM, Fura-2/acetyl-methoxy ester

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Their findings thus suggested ET-1 to be an endogenous modulator of myofibroblast-mediated granulation tissue contraction [8]. ET-1 is a potent vasoconstrictive peptide isolated from the culture medium of porcine vascular endothelial cells and one of a family of three isopeptides (ET-1, ET-2, and ET-3) [9,10]. Two subtypes of receptors, ET_A (selective for ET-1 and ET-2) and ET_B (non-selective) receptors, are known to mediate the responses made by ET-1 [11,12]. However, to our knowledge, there has been no report about which types of receptors for ET-1 exist on the myofibroblasts and mediate the contraction of granulation tissue. Although Appleton et al. demonstrated that ET-1 evoked contractions of granulation tissue could be partially blocked by use of calcium antagonists [8], the changes in the free Ca^{2+} concentration in the myofibroblasts caused by stimulation with ET-1 have not yet been fully elucidated.

In the present study, we examined the subtype of ET-1 receptors causing the contraction and the relationship between the intracellular Ca^{2+} mobilization and the contraction. We confirmed the existence of both ET_A and ET_B receptors on the croton oil-induced granulation tissue strip and myofibroblasts isolated from the tissue using the RT-PCR technique. We also examined the pharmacological properties of the ET-1 induced contraction of the granulation tissue pouch and the intracellular Ca^{2+} mobilization in the cultured myofibroblasts isolated from the pouch. These results suggest that the ET-1 induced contraction of granulation tissue is mainly mediated through the mobilization of Ca^{2+} from the extracellular space caused by the stimulation with ET_A receptors.

2. Materials and methods

2.1. Preparation of granulation tissue pouch

The granulation tissue pouch was prepared in male Wister rats (120–150 g) by the injection of first 30 ml of air into the dorsal subcutaneous tissue followed by 1 ml of 0.1% croton-oil solved in maize oil [8]. After 23–26 days, granulation tissue pouches were dissected and transferred to ice-cold Krebs–Henseleit solution containing (mM), NaCl 118.1, KCl 4.7, KH_2PO_4 1.17, $MgSO_4$ 2.41, $CaCl_2$ 5.05, D-glucose 11.1 and $NaHCO_3$ 25, bubbling with 95% O_2 and 5% CO_2.

2.2. Tension measurements

The granulation tissue pouches were cut into strips (5×10 mm) and incubated in an organ bath containing 20 ml of Krebs–Henseleit solution gassed with 95% O_2:5% CO_2 and maintained at 37°C at 1 g resting tension. After equilibration for 2 h, the strips were exposed to cumulative concentrations of ET-1 (10^{-10}–10^{-7} M) and IRL1620 (10^{-10}–10^{-6} M), and the tension development was measured using a type UL-20 GR transducer (Minebe). ET_A receptor selective antagonist BQ123 (10^{-6} M) or ET_B receptor selective antagonist BQ788 (10^{-7} M) was added to the organ bath 20 min before the addition of ET-1, and the ET-1 induced tension development was also measured in a Ca^{2+}-free solution containing 6 mM EGTA.

2.3. Cell culture

For the isolation of myofibrolasts, the granulation tissue pouch dissected from the rat dorsal subcutaneous tissue was rinsed in a sterile phosphate-buffered saline (PBS), minced finely, and then was digested in 20 ml Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 2000 U/ml dispase at 37°C for 1 h with a gentle stirring. The digestion was then terminated by centrifugation at 1000 rpm for 5 min at 4°C and the pellet was thereafter resuspended in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, and 100 U/ml streptomycin. These cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. For the isolation and culture of normal fibroblasts as a control sample, the connective tissue was dissected from the dorsal subcutaneous region of normal rats and was treated in the same way as described above. In this study, cells in passages 2–8 were used. There was no significant difference in the properties of the cultured cells examined among these passages.

2.4. Immunohistochemical staining

Immunohistochemical staining was performed using tissue sections of the aorta, granulation tissue pouch, back muscle, and back normal skin of rats. Paraffin-embedded sections were mounted onto glass slides and were washed in PBS. The sections were fixed with methanol and blocked with 10% rabbit serum for 10 min at room temperature, and then were incubated with mouse monoclonal anti-α-SM actin antibody for 45 min at room temperature. The sections were washed in PBS and incubated with rabbit anti-mouse IgG+IgA+IgM-biotin for 30 min. The slides were washed in PBS three times and incubated with streptavidin–alkaline phosphatase for 30 min. They were then treated with a histofine alkaline phosphate substrate kit. Slides were washed rapidly in PBS followed by washing with glycerol, coverslipped and then observed under a microscope.

2.5. Immunofluorescence staining

Myofibroblasts were plated on collagen-coated glass slides and grown in monolayer cultures. They were rinsed in PBS and fixed with acetone. The samples were blocked with 10% rabbit serum for 10 min at room temperature before probing with mouse monoclonal anti-α-SM actin antibody for 45 min at room temperature. Bound primary antibody was detected by incubating the slides for 30 min with rabbit anti-mouse IgG+IgA+IgM-biotin. They were then incubated with streptavidin–fluorescein isothiocyanate conjugate diluted 1:40 in PBS for 30 min, washed in PBS and glycerol, and then were coverslipped for the microscopic observation. We determined the percentage of myofibroblasts within the cultures by counting number of the cells which expressed bundles of α-SM actin.

2.6. Immunoblotting

Samples were separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) [13], and transferred to a polyvinylidene difluoride (PVDF) membrane using a semi-dry transfer system (1 h, 15 V). After blocking with 5% skim milk for 30 min, the membrane was probed with anti-α-SM actin antibody for 1 h at room temperature. The membrane was washed three times and incubated with rabbit × mouse IgG alkaline phosphatase for 20 min. Immunoreactive proteins were then visualized by treatment with an Alkaline Phosphatase Substrate Kit IV.

2.7. A PCR analysis of endothelin receptor mRNA

Total RNA from granulation tissue pouch or myofibroblasts was isolated by the acid guanidium thiocyanate/phenol/$CHCl_3$ method using Trizol reagent. Obtained total RNA (2 μg) was subjected to RT-PCR using Gene Amp RNA PCR Kit. The pair of primers used for ET_A receptor amplification were: sense primer, 5′-CTACGTGGGAATTCATCTCC-3′ (nucleotides 384–403); and antisense primer, 5′-AAGCCACGGATCCGTACCTG-3′ (nucleotides 546–565), which included *Eco*RI site or *Bam*HI site (underlined), respectively. Other pairs of primers used for ET_B receptor amplification were: sense primer, 5′-GGCTTCCCCTTGAGCTCAG-3′ (nucleotides 1004–1022); and antisense primer, 5′-GGAGCGGATCCTGTCGTAT-3′ (nucleotides 1285–1303), which included either the *Sac*I site or *Bam*HI site (underlined), respectively. The PCR products were subcloned into pUC119 vector for DNA sequencing to confirm the obtained fragments to be a part of the ET_A or ET_B receptors.

2.8. Measurement of the intracellular Ca^{2+}concentration ($[Ca^{2+}]_i$) in myofibroblasts

After harvesting the cells from confluent subcultures using 0.5% trypsin, the cells were suspended in a solution containing (mM), NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1.5, HEPES/Tris (pH 7.4) 10, glucose 10 and 0.1% bovine serum albumin, and plated onto coverslips (25 mm diameter). The cells were incubated with 6 μM Fura-2/acetyl-methoxy ester (Fura-2/ AM) for 1 h at 37°C, and mounted in a limited volume chamber on the stage of Axiovert 135 microscope (Carl Zeiss, Germany) equipped for Digital Fluorescence Microscopy Attofluor (Atto Instruments, USA). This chamber could be perfused by two polyethylene tubes for injection and suction attached to the edge of the chamber, and the stage was kept at 37°C. The Fura-2 fluorescence change of a single cell was monitored at 334 and 380 nm for excitation and 520 nm for emission, and $[Ca^{2+}]_i$ was shown by the fluorescence ratios (F_{334}/F_{380}). These experiments were also performed in a Ca^{2+}-

free solution containing (mM), NaCl 140, KCl 5, $MgCl_2$ 1, HEPES/Tris (pH 7.4) 10, glucose 10, EGTA 0.5 and 0.1% bovine serum albumin.

2.9. Materials

ET-1 and IRL1620 were purchased from the Peptide Institute (Osaka, Japan). BQ123 and BQ788 were kind gifts of Banyu (Ibaraki, Japan). Dispase was purchased from the Godo-Shyusei (Tokyo, Japan). Mouse monoclonal anti-α-SM actin antibody was purchased from Progen (Germany). Rabbit anti-mouse IgG+IgA+IgM-biotin, streptavidin–alkaline phosphatase, and a histofine alkaline phosphatase

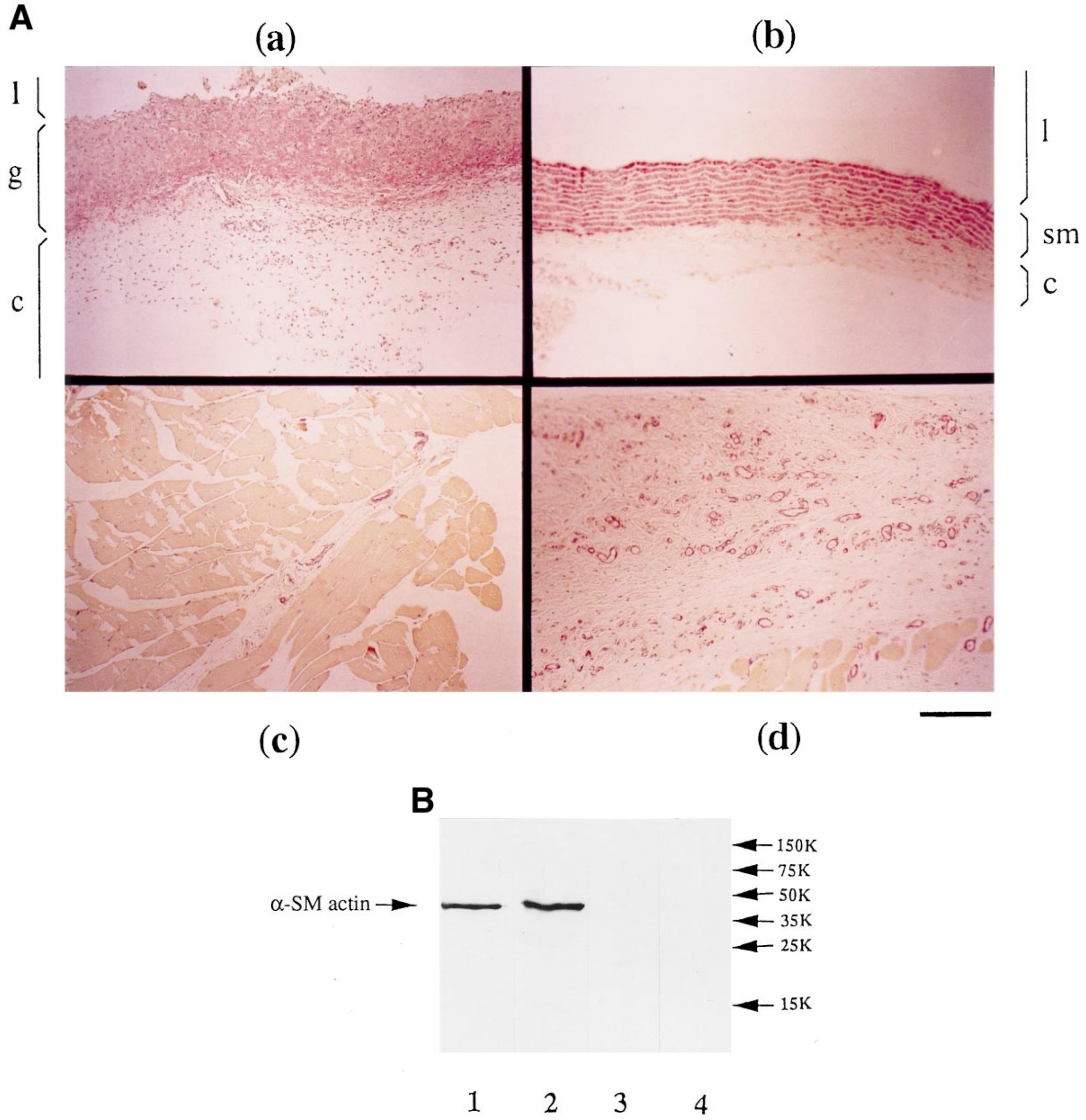

Fig. 1. Immunohistochemical staining and a Western blot analysis of rat tissue specimens with anti-α-SM actin antibody. (A) Immunohistochemical staining. Paraffin-embedded tissue sections of a granulation tissue pouch (23 days after croton oil injection), aorta, skeletal muscle (back muscle), and back skin were fixed with methanol and treated with anti-α-SM actin antibody, as described in Section 2. (a) granulation tissue pouch; (b) aorta; (c) skeletal muscle; (d) normal skin. l, lumen; g, granulation tissue; sm, smooth muscle layers; c, surrounding connective tissue. ×100. Scale bar: 50 μm. (B) Western blot analysis. The samples were dissolved in Laemmli's sample buffer and separated by 12% SDS-PAGE. Lane 1, granulation tissue pouch; lane 2, aorta; lane 3, skeletal muscle; lane 4, normal skin. The molecular weight is indicated in Da. The results are representative of three other experiments.

substrate kit were purchased from the Nichirei (Tokyo, Japan). Streptavidin–fluorescein isothiocyanate conjugate was from Gibco BRL. Rabbit × mouse IgG alkaline phosphatase was from Chemicon (USA). The Alkaline Phosphatase Substrate Kit IV was from Funakoshi (Tokyo, Japan). Trizol Reagent was from Gibco BRL. Gene Amp RNA PCR Kit was from Perkin Elmer (USA). Fura-2/AM was from Dojindo (Kumamoto, Japan).

3. Results

3.1. Immunochemical staining of the granulation tissue pouch with anti-α-SM actin antibody

As shown in Fig. 1Aa, anti-α-SM actin antibody stained intensely the fibroblast-like cells in the granulation tissue pouch formed in the rat dorsal subcutaneous region 23 days after the injection of croton oil. It was confirmed that the granulation tissue pouch was mostly composed of myofibroblasts, which are characterized by their rich α-SM actin [3–5,8]. In the skeletal muscle (back muscle) and the intact skin, however, only the wall of the microvessels scattered within the tissues were stained with anti-α-SM actin antibody (Fig. 1Ac,d). The smooth muscle layer of rat aorta was strongly stained with the anti-α-SM actin antibody (Fig. 1Ab). The presence of α-SM actin in the granulation tissue pouch and aorta was also shown by a Western blot analysis (Fig. 1B, lanes 1 and 2), whereas α-SM actin was hardly detected in the skeletal muscle or the intact skin (Fig. 1B, lanes 3 and 4). These findings were consistent with the results described above in the immunohistochemical staining.

3.2. Effects of BQ123, BQ788 and IRL1620 on the contraction of the granulation tissue pouch strips

Fig. 2 shows the tension record from the strips of the granulation tissue pouch. When ET-1 (10^{-10}–10^{-8} M) was applied cumulatively to the organ bath, tension developed in a dose-dependent manner and the half maximal tension was obtained at $2.04 \pm 0.29 \times 10^{-9}$ M (Fig. 2AFig. 3). The stable tension level at each concentration of ET-1 was preserved at the constant level at least half an hour and the tension declined gradually after ET-1 was washed out (Fig. 5).

In the presence of BQ123 (10^{-6} M), an ET_A receptor selective antagonist, both the threshold ET-1 concentration to elicit a contraction to the strip and the ET-1 concentration to develop the maximal tension are increased, and the half maximal tension was obtained at $19.4 \pm 5.3 \times 10^{-9}$ M, which was about 10 times higher than that in the absence of BQ123 (Fig. 2BFig. 3). The tension development by ET-1 was not significantly affected by the presence of BQ788 (10^{-7} M), an ET_B receptor selective antagonist (Fig. 2CFig. 3), and the half maximal tension was obtained at $1.93 \pm 0.27 \times 10^{-9}$ M ET-1. As shown in Fig. 2D, IRL1620 (10^{-9}–10^{-6} M), an ET_B receptor selective agonist, did not elicit any contraction, but the subsequent application of ET-1 did cause a contraction, whose dose–response profile was the same as that observed in the presence of ET-1 alone (see Fig. 2A).

3.3. Expression of ET_A and ET_B receptor subtypes in the granulation tissue pouch

In order to clarify which type of ET receptor is expressed in myofibroblasts, RT-PCR was carried out using the total RNA obtained from the granulation tissue pouch. The results clearly indicated the presence of both ET_A and ET_B receptor subtypes in the granulation tissue, and the former subtype also appeared to be more abundant (Fig. 4). From these results, it is most probable that the ET-1 induced contraction of the granulation tissue pouch was mainly mediated through ET_A receptor.

3.4. Effects of extracellular Ca^{2+} on the ET-1 induced contraction of the granulation tissue pouch strips

As shown in Fig. 5, the maximal contraction induced by ET-1 (10^{-7} M) in normal Krebs–Henseleit solution declined gradually after washing, but it returned to the resting level rapidly by adding 20 mM EGTA to a final concentration of 6 mM. By altering the solution to normal Krebs–Henseleit solution, the granulation tissue strip then developed some tension, which returned to the resting level again by adding EGTA to a final concentration of 6 mM.

3.5. Characterization of the cells isolated from the granulation tissue pouches

The cells from the granulation tissue pouches were isolated and cultured. Immunochemical staining and a Western blot analysis was carried out using anti-α-SM actin antibody to confirm the cultured cells isolated from granulation tissue pouches were myofibro-

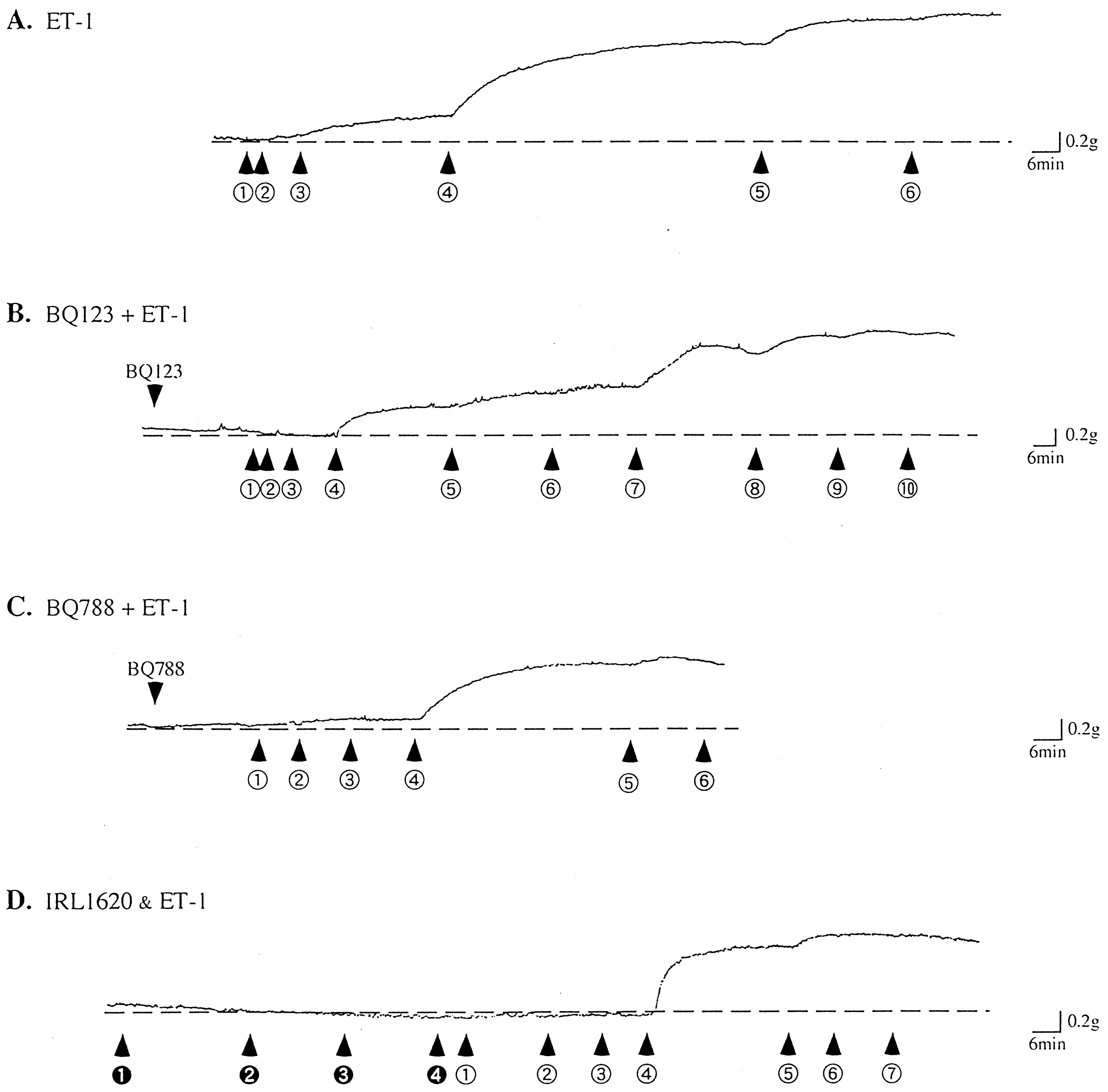

Fig. 2. Tension records from the strips of a granulation tissue pouch. ET-1 or IRL1620 was applied cumulatively in the bathing solution. Antagonists (BQ123 and BQ788) were applied 20 min before the application of ET-1. The traces are typical of three other experiments. ET-1, IRL1620 or antagonists were applied at the position of the arrow heads and the concentrations of the reagents were as follows. White-encircled numbers: 1–10, ET-1 (M); 1, 3×10^{-10}; 2, 7×10^{-10}; 3, 1×10^{-9}; 4, 3×10^{-9}; 5, 7×10^{-9}; 6, 1×10^{-8}; 7, 3×10^{-8}; 8, 5×10^{-8}; 9, 7×10^{-8}; and 10, 1×10^{-7}. Black-encircled numbers: 1–4, IRL1620 (M); 1, 1×10^{-9}; 2, 1×10^{-8}; 3, 1×10^{-7}; and 4, 1×10^{-6}. (A) ET-1 alone. (B) Pretreatment with 10^{-6} M BQ123. (C) Pretreatment with 10^{-7} M BQ788. (D) IRL1620 and ET-1.

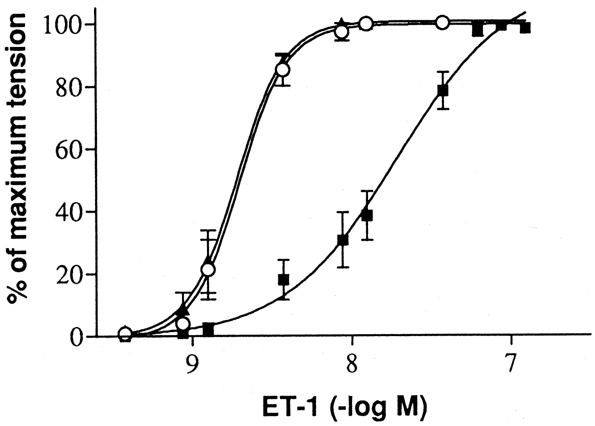

Fig. 3. Effects of BQ123 and BQ788 on the ET-1 induced contractions of a rat granulation tissue pouch strip. The concentration-dependent contraction curve to ET-1 in the absence of antagonists (○), and in the presence of BQ123 (10^{-6} M) (■) or BQ788 (10^{-7} M) (▲). Either antagonist was applied 20 min before the addition of ET-1. The data are relative to the maximum tension produced by ET-1 and are the mean ± S.E.M. from three experiments.

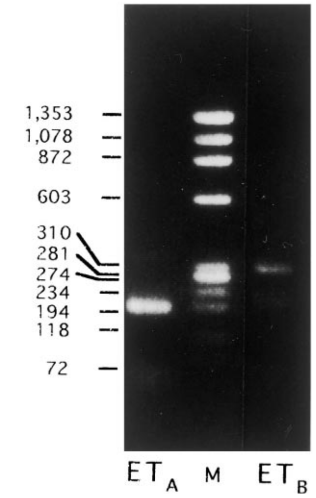

Fig. 4. mRNA expression of ET_A and ET_B receptors in the granulation tissue pouches. The total RNA (2 μg) from the granulation tissue was subjected to RT-PCR. The PCR products were electrophoresed on 2% agarose gel and then stained with ethidium bromide. ET_A, the expression of ET_A receptor mRNA; ET_B, the expression of ET_B receptor mRNA; M, DNA size marker, ϕX174-*Hae*II digest. The size of each band is given on the left of the photograph.

blasts. As shown in Fig. 6A, most cells isolated from granulation tissue pouches expressed bundles of α-SM actin, while the cultured fibroblasts isolated from rat intact dorsal subcutaneous region were stained only faintly by anti-α-SM actin antibody without forming bundles of α-SM actin (data not shown). The percentage of myofibroblasts in the cultured cells was 87.1 ± 0.95 (mean ± S.E.M., $n = 3$). This percentage did not significantly differ among cultures two to eight, which were used in this study. A Western blot analysis showed that both the granulation tissue pouch and the cells isolated from it were rich in α-SM actin, while a much smaller amount of α-SM actin was expressed in the fibroblasts isolated from the rat intact dorsal subcutaneous region (Fig. 6B). The expression of both ET_A and ET_B receptors were also confirmed in the cells isolated from granulation tissue pouches by RT-PCR (Fig. 6C). These results indicate that the cells isolated from the granulation tissue pouches show the same characteristics as the main component cells of the granulation tissue pouches, and they were thus proven to be myofibroblasts.

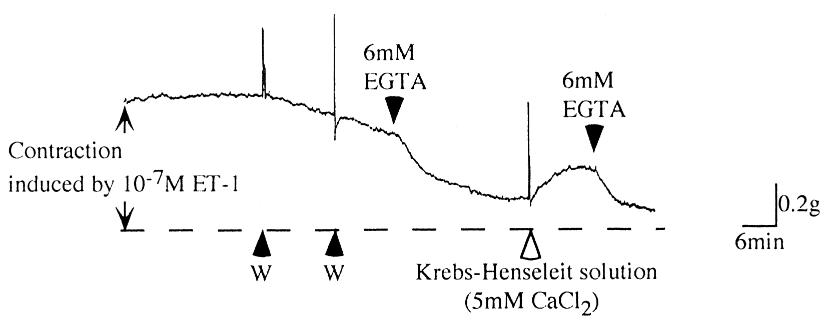

Fig. 5. Effect of Ca^{2+}-chelator (EGTA) on the ET-1-induced tension of a rat granulation tissue pouch strip. The strip was incubated in 20 ml of Krebs–Henseleit solution containing 5 mM Ca^{2+}, and 10^{-7} M ET-1 was applied to the solution. After ET-1 induced a maximum contraction on the strip, EGTA was applied at the position of the arrow heads. The trace is typical of three other experiments. W, wash.

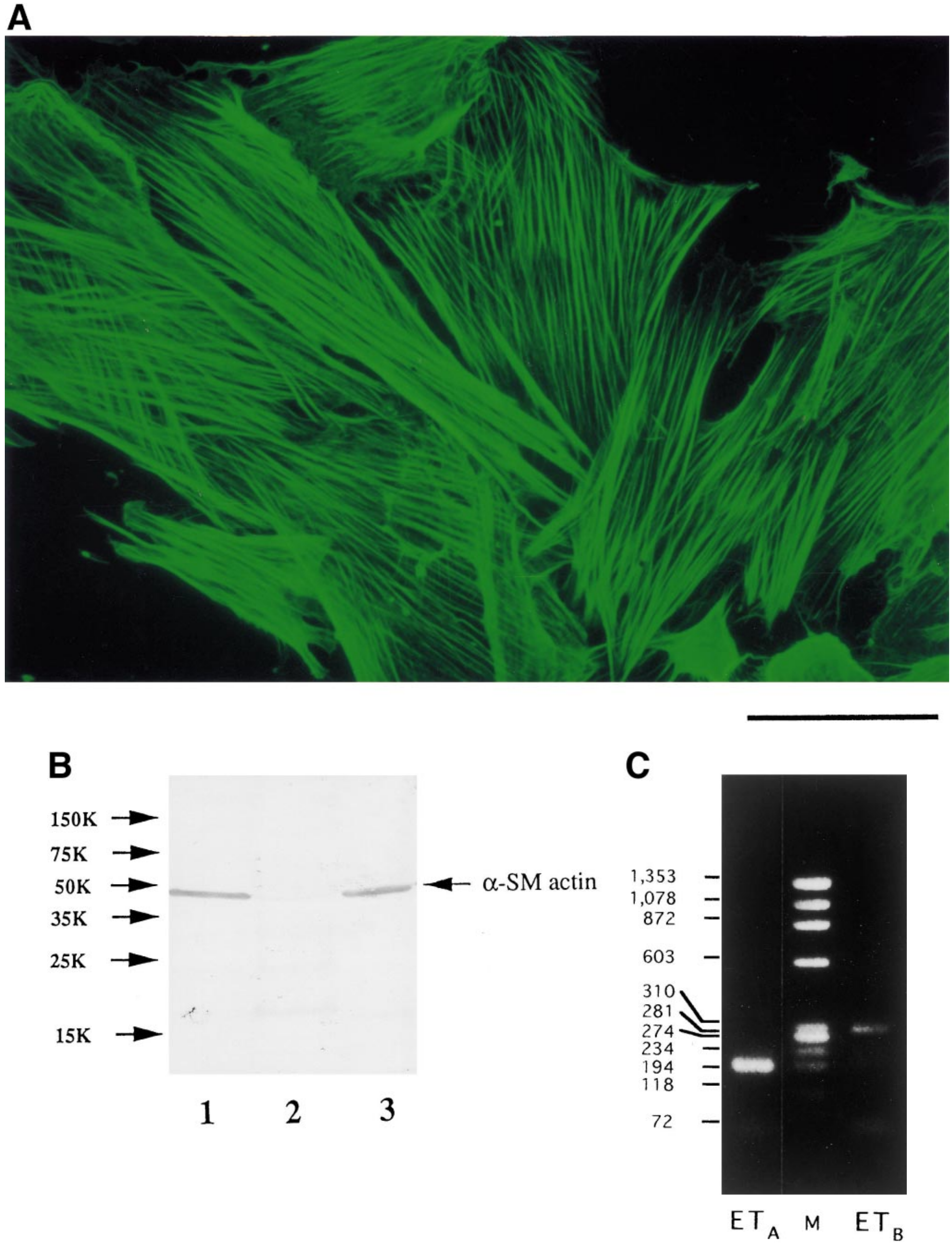

Fig. 6. Characterization of the cells isolated and cultured from the granulation tissue pouches. Each minced granulation tissue pouch was digested by dispase under sterilized conditions and the isolated cells were maintained in DMEM until they reached confluence. (A) Immunofluorescence staining with anti-α-SM actin antibody. The cells were plated and grown on collagen-coated glass-slides and fixed with acetone. The samples were probed with anti-α-SM actin antibody and bound antibody was visualized by fluorescein isothiocyanate, as described in Section 2. ×400. Scale bar: 20 μm. (B) Western blot analysis with anti-α-SM actin antibody. The samples were separated by 12% SDS-PAGE and immunoblotted for α-SM actin. Lane 1, cultured cells from a granulation tissue pouch; lane 2, cultured fibroblasts; lane 3, granulation tissue pouch. (C) mRNA expression of ET_A and ET_B receptor. Total RNA was extracted from the cultured cells and RT-PCR was carried out. The PCR products were electrophoresed on 2% agarose gel and stained with ethidium bromide. ET_A, the expression of ET_A receptor mRNA; ET_B, the expression of ET_B receptor mRNA; M, DNA size marker, ϕX174-*Hae*II digest. The size of each band is given on the left of the photograph.

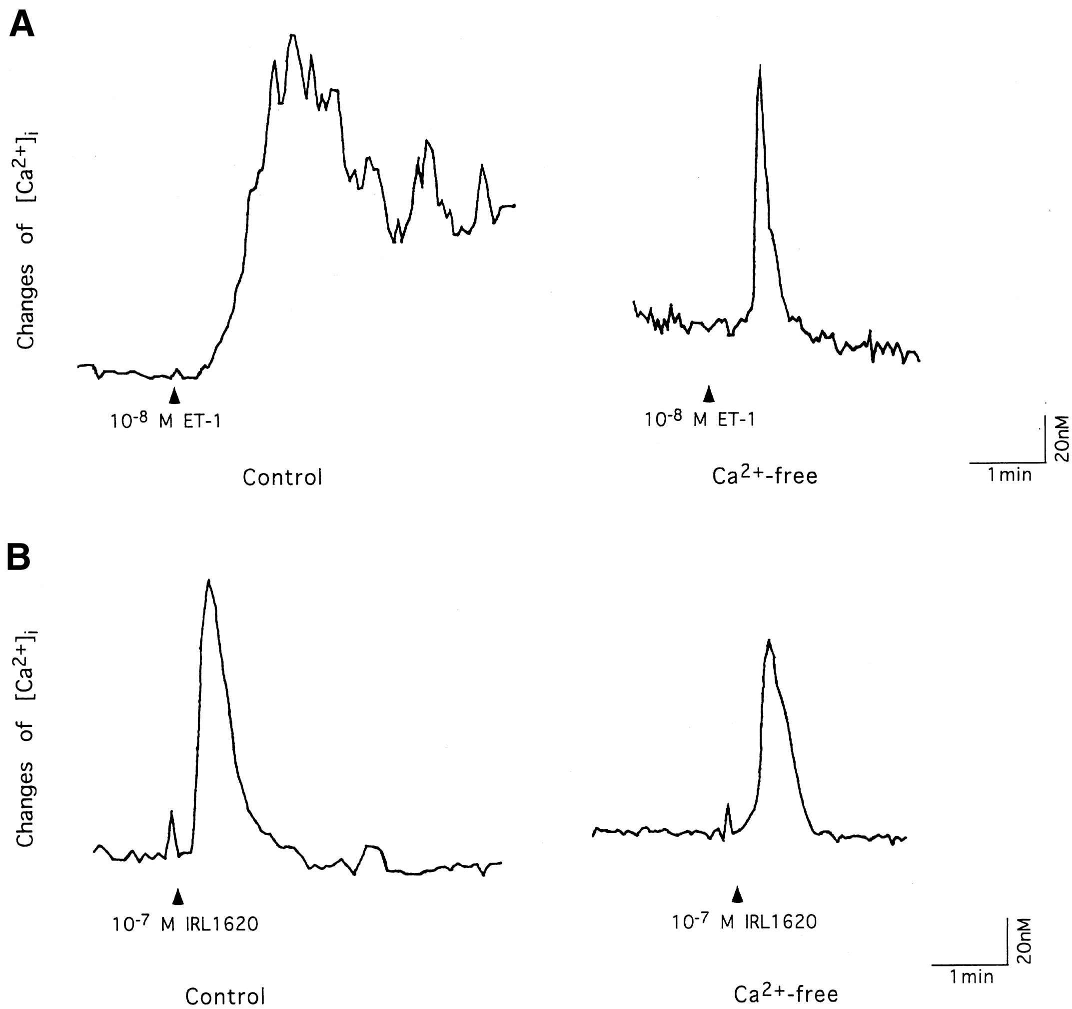

Fig. 7. Effect of ET-1 and IRL1620 on $[Ca^{2+}]_i$ in the cultured myofibroblasts. The changes of $[Ca^{2+}]_i$ induced by 10^{-8} M ET-1 (A) or 10^{-7} M IRL1620 (B) in Fura-2 loaded myofibroblasts were monitored in the presence (left) or absence (right) of extracellular Ca^{2+} (1.5 mM). The traces are typical of five other experiments.

3.6. Intracellular Ca^{2+} mobilization of myofibroblasts

To investigate the relationship between the contraction of granulation tissue pouch and the intracellular Ca^{2+} mobilization in myofibroblasts induced by ET-1, $[Ca^{2+}]_i$ was measured with the ratiometric fluorescence dye Fura-2/AM in each cultured myofibroblast. ET-1 (10^{-8} M) produced a transient increase in $[Ca^{2+}]_i$ followed by a sustained oscillatory elevation of $[Ca^{2+}]_i$ (Fig. 7A). The duration of the sustained elevation of $[Ca^{2+}]_i$ varied, ranging from 2–3 to 60 min. In the Ca^{2+}-free solution, a sustained increase in $[Ca^{2+}]_i$ caused by ET-1 was hardly observed (Fig. 7A). The cultured myofibroblasts also showed the transient increase in $[Ca^{2+}]_i$ by IRL1620 (10^{-7} M), in the presence and absence of Ca^{2+} in the bathing solution, but no sustained elevation of $[Ca^{2+}]_i$ was observed in either situation (Fig. 7B). Furthermore, the effects of BQ123 and BQ788 on the intracellular Ca^{2+} mobilization in the myofibroblasts induced by ET-1 were examined. The number of the cells which responded to ET-1 (10^{-8} M) decreased in the presence of BQ123 (10^{-6} M), but it remained unchanged in the presence of BQ788 (10^{-7} M). The number of the cells which responded to IRL1620 (10^{-7} M) decreased in the presence of BQ788 (10^{-7} M). However, in the experiments using ET receptor antagonists, the responses to ET-1 and IRL1620 varied too much to identify which cells were actually antagonized or damaged in the process of incubation (data not shown). These results indicated that the sustained elevation of $[Ca^{2+}]_i$ was due to an influx from extracellular Ca^{2+} and ET-1 caused the influx of extracellular Ca^{2+} via the activation of ET_A receptors. Freshly prepared myofibroblasts (within 6 h after isolation) showed essentially the same properties as the cultured myofibroblasts after six to eight passages (data not shown).

4. Discussion

The granulation tissue which forms during the process of wound healing is known to be mainly composed of myofibroblasts, which is a contractile phenotype of fibroblasts and contains a lot of cytoskeletal proteins such as α-SM actin in the cytoplasm [3–5].

In the present study, a granulation tissue pouch was prepared in the dorsal subcutaneous tissue by injecting a rat with croton oil which is an irritative. Strips of granulation tissue pouch have been reported to show active tension development by the application of several pharmacological reagents [6–8]. Since the contents of ET-1 synthesized endogenously in the granulation tissue have been reported to correlate well with the extent of contraction or shrinkage of the wound [8], we focused our attention on the pharmacological properties of the contraction of the granulation tissue pouch caused by ET-1.

Endothelins are a family of three homologous peptides (ET-1, ET-2 and ET-3) that display a wide variety of biological activities [10]. Two subtypes of receptors, ET_A and ET_B receptors, are known to mediate the responses made by endothelins in mammals. The ET_A receptor binds ET-1 with a higher affinity than ET-2 and ET-3, while the ET_B receptor displays an equal affinity for all ET peptides [11,12,14]. We demonstrated, using the RT-PCR technique, that both ET_A and ET_B receptors exist in the croton oil induced granulation tissue pouch and myofibroblasts isolated from the pouch. As shown in Figs. 4 and 6C, the relative expression of ET_A receptor was much higher than that of the ET_B receptor. However, a quantitative analysis of the gene expression using the RT-PCR technique is unreliable because the amount of the PCR product increases exponentially with each cycle of amplification. Furthermore, we observed the opposite results when we used another pair of primers for amplification (data not shown). Therefore, we could not estimate the relative ratio of the amount of these receptors from the results obtained using the PCR technique, but it was observed that a much smaller number of cells showed an increase of $[Ca^{2+}]_i$ by IRL1620 than by ET-1, thus suggesting that the ET_B receptor is expressed to a lesser degree than the ET_A receptor in croton oil-induced granulation tissue.

We observed ET-1 to induce a concentration-dependent contraction in the granulation tissue pouch. The pretreatment with BQ123, an ET_A receptor selective antagonist, shifted the concentration–response curve to the right, but the pretreatment with BQ788, an ET_B receptor selective antagonist showed little effect. Furthermore, IRL1620, the ET_B receptor selective agonist, did not cause contraction. As shown in Fig. 5, the chelation of Ca^{2+} in the Krebs solution by EGTA (6 mM) promptly decreased the tension development almost to a resting level and the subsequent replacement of the bathing solution with the normal Krebs solution containing 5 mM Ca^{2+} helped the active tension to recover. This result indicated that the influx of extracellular Ca^{2+} was necessary for the ET-1 induced contraction of the granulation tissue strip. There have been several studies reporting the transient increase in $[Ca^{2+}]_i$ to not be related to the contraction, and instead, the subsequent increase in $[Ca^{2+}]_i$ produced by influx from extracellular Ca^{2+} through non voltage dependent Ca^{2+} channels caused a sustained contraction in several kinds of tissue, i.e. rat aorta, porcine coronary artery, trachea, rabbit iris dilator muscle and aorta [15–19]. Considering the effects of ET receptor antagonists on the ET-1 induced contractile response and the fact that the sustained elevation of $[Ca^{2+}]_i$ was produced only by ET-1 (but not by IRL1620) in the presence of extracellular Ca^{2+}, it is thus suggested that the contraction of the granulation tissue pouch caused by ET-1 is mainly mediated by ET_A receptors and requires influx of extracellular Ca^{2+}. Although no contractile response was caused by the activation of ET_B receptor with IRL1620, a transient increase of $[Ca^{2+}]_i$ was observed in the myofibroblasts. These results possibly indicate that the activation of ET_B receptor in myofibroblasts might thus induce some other biological effect than contraction or relaxation. However, this hypothesis still remains to be elucidated.

The circulating and local levels of ET-1 are reported to be elevated during the process of wound healing [20], and wounds topically treated with ET-1 are also known to heal quickly [21]. ET-1 antibodies have also been reported to block wound contraction in healing skin while endogenous ET-1 within the wound modulates the contractile effects of myofibroblasts [22]. Our observation that the contraction caused by ET-1 was long lasting may help wound shrinkage. ET-1 promotes cell proliferation in a variety of cell types including vascular smooth muscle cells [23,24] and fibroblasts [25]. Although the precise mechanisms of wound healing and granulation tissue contraction remain to be clarified, ET-1 seems to be a promoter of this process, mainly by the contraction of myofibrobasts. The above findings may thus contribute to the clinical therapeutic benefit of the wound healing process. Further in vivo as well as in vitro studies are required to confirm this hypothesis.

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